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Self-Assembly of Hydrogels From Elastin-Mimetic Block Copolymers

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ABSTRACT

Triblock copolymers have traditionally been synthesized with conventional organic components. However, triblock copolymers could be synthesized by the incorporation of two incompatible protein-based polymers. The polypeptides would differ in their hydrophobicity and confer unique physiochemical properties to the resultant materials. One protein-based polymer, based on a sequence of native elastin, that has been utilized in the synthesis of biomaterials is poly (Valine-Proline-Glycine-Valine-Glycine) or poly(VPGVG) [1]. This polypeptide has been shown to have an inverse temperature transition that can be adjusted by non-conservative amino acid substitutions in the fourth position [2]. By combining polypeptide blocks with different inverse temperature transition values due to hydrophobicity differences, we expect to produce amphiphilic polypeptides capable of self-assembly into hydrogels. Our research examines the design, synthesis and characterization of elastin-mimetic block copolymers as functional biomaterials. The methods that are used for the characterization include variable temperature 1D and 2D High-Resolution-NMR, cryo-High Resolutions Scanning Electron Microscopy and Differential Scanning Calorimetry.

INTRODUCTION

Biomaterials have become increasingly important in medical research and applied medicine. They are used in many roles, from drug delivery systems to implantation devices. Biomaterials are varied in their composition; they can be composed of synthetic organic polymers, ceramics, metals and protein-based polymers. Protein-based polymers are of interest because they do not have some of the biocompatibility issues of some other materials; this is attributable to their similarity to native protein materials [3]. The focus of this research is the synthesis of amphiphilic block copolymers based on elastin-mimetic peptide sequences. These amphiphilic block copolymers undergo heat-induced micellization and form complex fluids in aqueous solution [4]. Elastin-mimetics are of special interest in medical applications because they do not adhere to cells and are non-immunogenic. We have designed blocks that, when combined, would provide different physiochemical properties to the copolymers. The peptides are modifications of the naturally occurring vertebrate elastin sequence, Valine-Proline-Glycine-Valine-Glycine (VPGVG). The peptide sequences will be joined together sequentially to form diblock and triblock copolymers. The block copolymers are being produced to afford unique

micelle- and gel-forming proteins. The resulting proteins could be used as the basis of drug delivery systems, coatings for implantation devices, or other medical roles.

EXPERIMENTAL DETAILS

All reagents were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Corporation (St. Louis, MO). Enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Plasmids pZErO 1.1, pZErO 2.0 and E. coli strain Top10F' were from Invitrogen Corporation (Carlsbad, CA). E. coli strains SCS110 and BL21Gold(DE3) were obtained from Stratagene (La Jolla, CA). Plasmid purification kits were purchased from Qiagen Inc. (Chatsworth, CA). Genosys Biotechnologies, Inc. (The Woodlands, TX) synthesized all single-stranded DNA oligonucleotides. Reagents were sterilized by either autoclave or passage through a 0.22 μm filter.

Procedures for DNA manipulations, transformation of competent cells, and the growth of bacterial cultures were adapted from literature [5] or the instructions supplied by manufacturers. Enzymatic reactions were performed in the buffers supplied by the manufacturers. An Applied Biosystems Voyager-DE™ STR Biospectrometry™ matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) was utilized to determine mass of the proteins. All the proteins, which have been expressed and purified, were run on 10-15 % gradient SDS-PAGE (Pharmacia Biotech PHAST System) with a negative zinc-staining technique in order to determine molecular weight and purity.

All solution NMR experiments were carried out with samples consisting of 43 mg of protein and 1 mg of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard (0.0 ppm), dissolved in 50:50 sterile ddH_2O and D_2O. Spectra were recorded on a Varian INOVA 600 (600 MHz, ^1H; 150 MHz ^{13}C) instrument. Samples were run at 4 °C and 25 °C to follow the changes in the copolymers' structure due to hydrophobic collapse of the plastin end-blocks. Standard solvent suppression techniques were employed to reduce signal due to the residual protons of water in the ^1H NMR of aqueous solutions. The HMQC NMR experiments were acquired at 4 °C and 25 °C with a 90° pulse of 8 μs on the proton (sweep width 5500.2 Hz) and a 90° pulse of 12 μs on the carbon. The data matrix contained 256 t_1 increments (sweep width in F_1 (carbon), 33 999.2 Hz) at 96 scans per increment. The NMR data were processed using the program NutsPro from Acorn NMR, Inc. (Livermore, CA).

Differential scanning Calorimetry (DSC) was performed by Dr. Alan Cooper, Chemistry Dept., University of Glasgow, Scotland, UK using a Microcal VP-DSC instrument, as detailed previously [6, 7]. The temperature range was 5 ° to 60 °C at a scan rate of 1 °C/min. The sample concentrations were approximately 1 mg/ml. Sample pH was varied between high and low pH.

Approximately 5 to 10 μL of the solutions were pipetted into 3 mm gold planchets (Balzers BU 012 130T), which had been pre-equilibrated to 4 °C in an isothermal environmental cooler (Torrey Pines Scientific). The temperature of the cooler was raised to 20 to 25 °C and allowed to stabilize for 10 minutes. The solidified samples were plunge frozen in liquid ethane at its melting point (-183°C); the samples were stored in liquid nitrogen (LN_2). A sample was transferred to and mounted on the precooled (~-170 °C) Oxford CT-3500 cryo-stage held in the cryo-preparation chamber. The specimen

was fractured with a prechilled blade and washed with LN_2. The shutters on the stage were closed to minimize frost contamination and the cryo-stage was transferred to the Denton DV-602 Cr coater. At this point, if the samples were to be etched in order to remove excess water (vitreous ice), the stage was allowed to equilibrate in a vacuum of ~10^{-7} torr. Once this occurred, the shutters were opened and the stage was brought to a temperature between –105°C and –99°C for varying time intervals. The stage shutters were closed and the stage was returned to ~-170°C. A 1 nm film was sputter coated onto the specimen, the stage shutters were closed and the stage was transferred to the upper stage of the DS-130F field emission scanning electron microscope operated at 25 kV. During the imaging process, specimen temperature was maintained at –115 °C. Images were digitally collected (5 Mbytes) in 16 s in order to reduce radiation damage.

DISCUSSION

Synthetic gene construction

The methods used to produce the DNA inserts which encode the various elastin block copolymers has been described elsewhere [8]. The basic scheme follows. Single-stranded DNA was annealed together to form double-stranded DNA with cohesive ends. The double-stranded DNA 'monomers' were inserted into plasmids engineered for DNA modifications (pZErO 1.1 and pZErO 2). Once monomers were found which had the correct sequence, the plasmid was purified on a large scale and the monomers were removed via restriction enzyme digestion. The resulting monomers were concatemerized to produce large repetitive genes (1200 to 3000 base pairs), which were enzymatically ligated into a modified expression plasmid (pET 24a) to afford the block copolymers.

Protein expression and purification

Expression plasmids containing the DNA sequences encoding proteins of suitable size and correct sequence were transformed into E. coli strain BL21(Gold)DE3. The proteins were expressed and purified following a hyperexpression protocol defined by Daniell et. al. [9]. This method allowed the production of gram quantities of the proteins of interest. Depending on the characteristics of each polymer, modifications to the purification protocol were made in order to afford the greatest quantity of pure protein.

The amino acid sequences for two of the resulting polypeptides are defined in Table I. Protein 1 is a diblock consisting of two equivalent polypeptides. Protein 2 is a triblock of two equivalent end-blocks and a unique internal block. From SDS-PAGE analysis, the estimated size of protein **1** is 80 kDaltons and protein **2** is 150 kDaltons. Amino acid compositional analysis, **1**; Calc. [mol-%]: Ala, 19.8; Gly, 20.2; Ile, 15.7; Pro, 17.66; Val, 24.3; Obs. [mol-%]: Ala, 19.7; Gly, 20.1; Ile, 14.9; Pro, 21.6; Val, 24.3; **2**; Calc. [mol-%]: Ala, 10.4; Glx, 1.9; Gly, 29.6; Ile, 8.2; Pro, 20.0; Val, 29.9; Obs. [mol-%]: Ala, 11.14; Glx, 2.9; Gly, 29.62; Ile, 9.64; Pro, 17.66; Val, 29.51 and MALD-TOF MS, Obs. (Calc): **1**, 72 016 (72 116); **2**, 134 097 (134 438), confirmed the identity of the recombinant polypeptides.

Table I. Amino acid sequences for the two elastin-mimetic block copolymers.
{VPAVG[(IPAVG)$_4$(VPAVG)]$_{16}$IPAVG}-[X]-{VPAVG[(IPAVG)$_4$(VPAVG)]$_{16}$IPAVG}
1; [X] = VPGVGVPGVG
2; [X] = VPGVG[(VPGVG)$_2$VPGEG(VPGVG)$_2$]$_{30}$VPGVG

(A, Alanine; G, Glycine; I, Isoleucine; P, Proline; V, Valine)

Copolymer characterization

On analysis of the ^{1}H, ^{13}C, and HMQC spectra, we determined that the resonances associated with the plastin end-blocks disappear at 25 °C due to their hydrophobic collapse above the inverse temperature transition. Resonances that noticeably disappear when comparing the (CH)α region of the ^{1}H, ^{13}C HMQC NMR spectra of protein **2** (Figure 1) are those associated with alanine and isoleucine contained in the end-blocks of the polypeptide. Individual resonances were assigned from known chemical shifts of elastin-mimetic polymer sequences [10]. The remaining resonances were assigned to the end-blocks and are commensurate with literature values [11, 12].

The DSC data obtained for the block copolymers shows a single transition between 21 °C and 23 °C. The transitions were reversible and minutely dependent on sample pH. Representative data for protein **2** is presented in Figure 2.

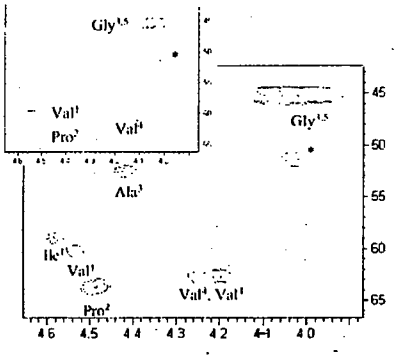

Figure 1. HMQC NMR data for triblock protein **2** at 4 °C and 25 °C (inset). Note the absence of resonances, which are assigned to the endblocks at 25 °C (inset).

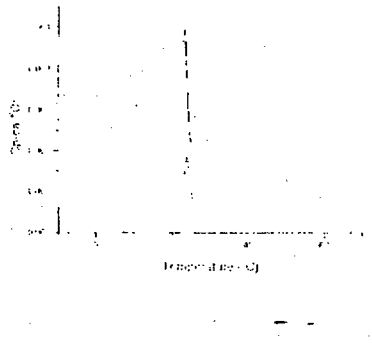

Figure 2. DSC data for the triblock protein **2**; demonstrating the inverse temperature transition lies at approximately 23 °C and is independent of pH

Analysis of the data from the cryo-HRSEM studies reveals strong correlations to elastin-mimetic hydrogels produced by chemical crosslinking [13]. Electron micrographs of protein **2** are in Figure 3. The average size of the micelles for protein **2** ranged from 15

to 20 nm (A). After etching (B), protein 2 displayed micelles of 15 to 20 nm in diameter connected by fibrils of less than 10 nm in diameter. The micelles are evenly spaced along the lengths of the fibrils. This feature was attributed to the size of the midblock of protein 2 limiting the aggregation behavior of the endblock domains.

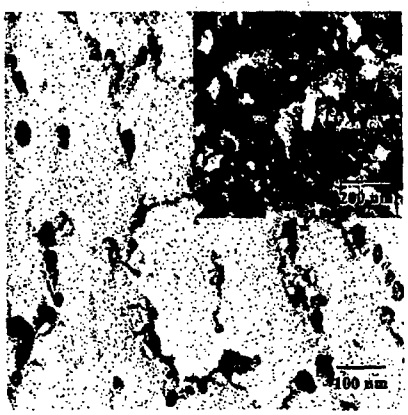

Figure 3. Cryo-HRSEM images of vitrified specimens of a concentrated aqueous solution of protein 2 prepared via rapid cryo-immobilization from gel specimen equilibrated at 25 C. Inset, specimen etched prior to image collection by controlled sublimation of vitreous ice to expose protein at interface.

CONCLUSIONS

The data presented demonstrates our ability to design and synthesize elastin-mimetic triblock copolymers that are capable of thermoreversible self-assembly into hydrogels. The characterization of the copolymers supports the hypothesis that the copolymer systems consist of a network of interconnected micelles of the end-blocks at increased concentrations (20 to 30 % w/v) when raised above the inverse transition temperature of the polypeptide.

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